

Callus induction and plant regeneration via leaf segments of three accessions of African rice (*Oryza glaberrima* Steud.)

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Abstract— A study conducted with the aim of developing a protocol for callus induction and plantlet regeneration *in vitro* from leaf segments of three accessions of African rice (*O. glaberrima* Steud.) indigenous to Ghana. Leaf segments of the accessions namely, Guame, N/4 and SARI 1 were assessed for callus induction and plantlet regeneration ability on different concentrations of plant growth regulators, incorporated into Murashige and Skoog, (1962) (MS) basal medium. Frequency of callus induction which was achieved on MS medium supplemented with (0-10) mg/l 2,4-D differed significantly ($p \leq 0.05$) among the accessions, as well as among the levels of 2,4-dichlorophenoxyacetic acid (2,4-D) tested. Highest callus induction frequency was exhibited at a concentration of 6 mg/l 2,4-D for all three accessions. Sub-culturing of callus on regeneration medium, which consisted of MS supplemented with (1:0-5) mg/l NAA:BAP resulted in no plantlet regeneration in all tested accessions. Instead, prolific root formation was observed.

Keywords— callus induction, *Oryza glaberrima*, picloram, plantlet regeneration and 2,4-D.

I. INTRODUCTION

Rice is the most important food crop in the world and feeds over half of the global population (Sasaki, 2005). Increased production of the commodity is necessary to meet the predicted demands of an ever increasing population. One option is to increase the area under rice cultivation, which is getting more difficult as more farmlands are being converted to residential areas in the developing world. The most viable option, therefore, is to increase productivity by advances in biotechnology (Bajaj and Mohanty, 2005).

Biotechnological techniques have been used to improve the existing cultivars, for the synthesis of novel plants and early release of high-yielding plants and plants resistant to

various diseases, pests, stresses and temperature. The successful application of technology for crop improvement requires suitable *in vitro* plant regeneration methods. Callus, which is an unorganised, proliferative mass of differentiated plant cells, is one of such means by which crop improvement can be undertaken. The ability to regenerate plants from callus is influenced by physiological factors as well as the genotype of the plant (Henry *et al.*, 1994). The regeneration of plants of some cereal crops such as bread wheat (Redway *et al.*, 1990; Vasile *et al.*, 1990), barley (Luhns and Lorz, 1987), rice (Yamada *et al.*, 1986) and maize (Duncan *et al.*, 1985), from callus have been documented.

In rice, there are reports on successful plant regeneration from explants such as coleoptile (Oinam and Kothari, 1995), root tips (Sticklen, 1991), immature embryos (Koetje *et al.*, 1989), leaf blades (Yan and Zhao, 1982), and other parts of *O. sativa*. However, protocol for callus induction and plant regeneration for *O. glaberrima* accessions have not been achieved. The objective of this study was to induce callus and regenerate plantlets *in vitro* in three different accessions of *O. glaberrima* by determining the hormone types and their concentrations suitable for inducing callus from their leaf segments as well as determining the hormone types and hormone concentrations /combinations for regenerating plants from leaf-derived calli of three accessions of *O. glaberrima*.

II. MATERIALS AND METHODS

2.1 Callus induction from leaf segments of *O. glaberrima*.

Seeds of three *Oryza glaberrima* accessions namely N/4, Guame and SARI 1 were manually dehusked and were surface sterilized by immersing in 0.1% mercuric chloride (HgCl₂) and vigorously agitated for 2 minutes under the

laminar flow hood and thereafter rinsed with three changes of sterile distilled water. The sterilized seeds were inoculated in test tubes containing 1.15ml of hormone-free Murashige and Skoog (MS) (1962) basal medium prepared from stock, supplemented with 30 g/l sucrose and 100 mg/l myo-inositol with pH 5.8 adjusted using 1M KOH prior to addition of 3.5 g/l phytigel and autoclaving at 121°C for 15 minutes at 15 psi. The cultures were kept in a growth room at a temperature of 21°C under a 16/8-hr (light/dark) photoperiod with light provided by white fluorescent tubes (T 5 fluorescent fitting, UK) at an intensity of 3000 lux. Seeds were allowed to germinate under these conditions to produce seedlings. Leaves obtained from four-days old *in vitro* germinated *O. glaberrima* seedlings were excised from base and cut into three pieces (referred to as segments 1-3, with 1 being the leaf base segment closest to the seed and 3 being the segment closest to the tip of the leaf) and inoculated in culture jars containing MS medium supplemented with different concentrations of picloram (0-10 mg/l) or 2,4-dichlorophenoxyacetic acid (2,4-D) (0-10 mg/l) together with 30 g/l sucrose and 100 mg/l myo-inositol. The pH was adjusted to 5.8 and 3.5 g/l phytigel added to the medium before autoclaving at 121°C for 15 minutes at 15 psi. The cultures were subsequently incubated in total darkness at 21°C for 12 weeks.

The experiment was set up as a completely randomized factorial design. The factors tested were three accessions of *O. glaberrima* by six concentrations of 2,4-D by six concentrations of picloram. Data were subjected to analysis of variance (ANOVA) based on 5 replications for percentage callus formed. The means were separated, where appropriate, at the 5% significance level using the least significant difference (LSD). Data were analyzed using Genstat statistical package.

2.2 Plant regeneration from leaf-derived callus of *O. glaberrima*

Calli obtained were sub-cultured on MS medium supplemented with naphthaleneacetic acid (NAA) and 6-

benzylaminopurine (BAP) in a ratio of (1:0-5) mg/l NAA:BAP followed by addition of 30 g/l sucrose and 100 mg/l myo-inositol. The pH was adjusted to 5.8 and 3.5 g/l phytigel added to the medium before autoclaving at 121°C for 15 minutes at 15 psi. The cultures were subsequently kept in a growth room at a temperature of 21°C under a 16/8-hr (light/dark) photoperiod with light provided by white fluorescent tubes (T 5 fluorescent fitting, UK) at an intensity of 3000 lux.

The experiment was set up as a completely randomized factorial design, (3x6). The factors tested were three accessions of *O. glaberrima* by six concentrations of NAA:BAP.

III. RESULTS

3.1 Effect of concentration of 2,4-D or picloram on callus formation from leaf segments of *O. glaberrima*

Leaf segment explants of the three *O. glaberrima* accessions; N/4, Guame and SARI 1 cultured on MS medium supplemented with varying concentrations (0-10 mg/l) of picloram did not develop callus irrespective of the accession or concentration of picloram present in the medium. However there was development of callus from leaf segment explants cultured on the same MS medium but supplemented with varying concentrations of 2,4-D (0-10) mg/l, occurred after 12 weeks of culture.

Amongst the three portions of leaf segments inoculated, only segment 1 (leaf base) was tissue culture responsive (Fig.1(D)). Segments 2 (middle) and 3 (tip) did not form callus on any of the callus induction media tried. All calli obtained from this experiment were formed from the leaf bases (segment 1).

Colour of callus ranged from cream (Fig.1(E)) to pale yellow (Fig.1(F)) to brown with callus becoming intense in colour as concentration of auxin in medium increased, eventually becoming necrotic at 8mg/l (Fig.1(G)). Higher concentrations of 2,4-D caused necrosis of the initiated calli.

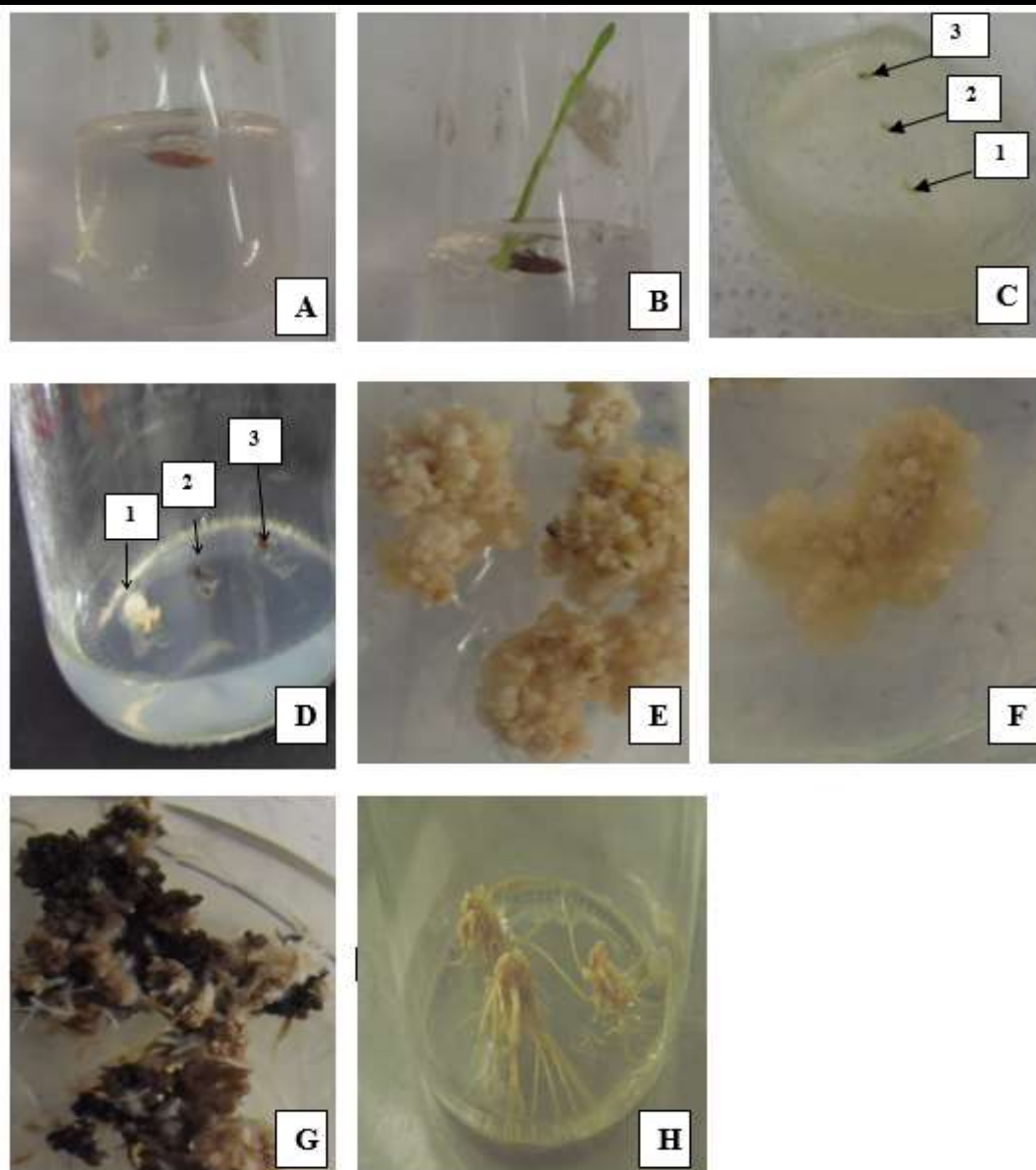


Fig.1 Callus induction from leaf segment explants of *O. glaberrima* seedling

(A) Dehusked mature seed inoculated on hormone-free medium; (B) 4 days old in vitro germinated *O. glaberrima* seedling; (C) Leaf segments inoculated on callus induction medium; (D) Callus formed from leaf base (segment 1); [(E-G) Callus formed from the leaf base (segment 1) showing different colours; (E) Cream (F) Pale yellow (G) Necrotic calli]; (H) Calli showing only root formation

Callus formation was observed in all the three rice accessions following addition of 4 mg/l, 6 mg/l and 8 mg/l 2,4-D to the culture medium (Fig.2). The highest percentage callus formation was recorded at 6.0 mg/l 2,4-D, recorded in all the three rice accessions, and which proved significantly higher compared to percentage callus formation at other concentrations of 2,4-D used, in each case. Percentage callus formation following an increase in

concentration of 2,4-D from 6 mg/l to 8 mg/l was not statistically different ($p \geq 0.05$) from a decrease in 2,4-D concentration from 6 mg/l to 4 mg/l (Fig.2). Callus formation however, depended on the concentration of the auxinin the culture medium. The percentage callus formation increased as the concentration of 2,4-D in the media increased for all the *O. glaberrima* accessions used.

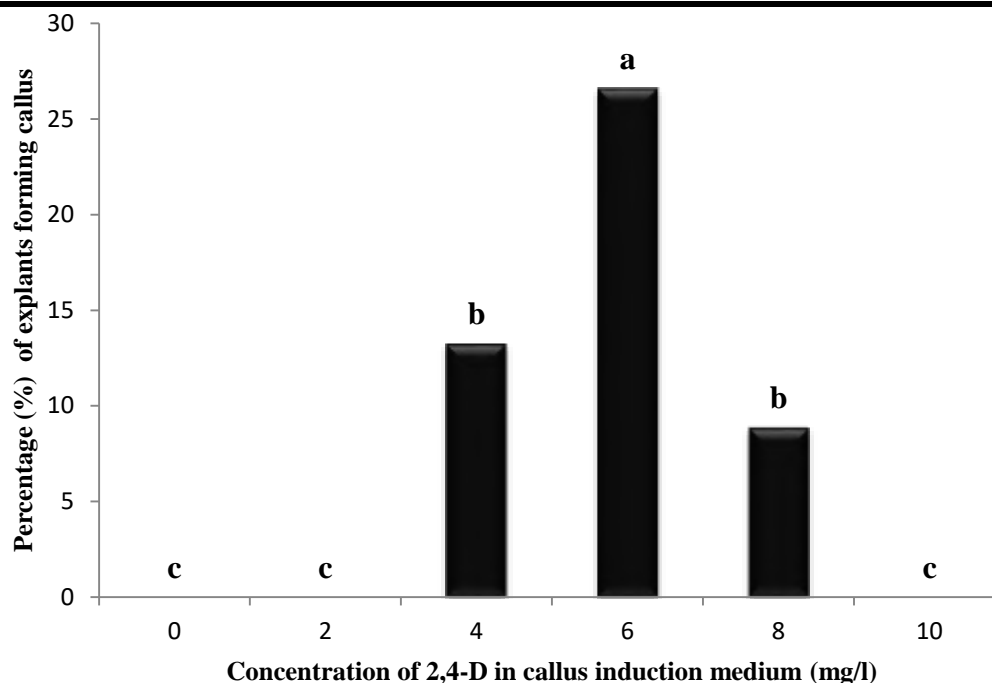


Fig.2: Effect of increasing concentration of 2,4-D in callus induction medium on percentage callus formation (Bars having the same letter are not significantly different ($P \geq 0.05$)).

3.2 Effect of accession of *O. glaberrima* on callus formation from leaf segments

Callus induction from *O. glaberrima* used for this study was found to be variable and accession dependent. Among the three accessions, N/4 recorded the highest number of explants forming callus (12.2%) from inoculated leaf

segments, which was significantly ($p \leq 0.05$) higher than the other two accessions (Fig.3). Frequencies of callus induction from the leaf segments of Guame and SARI 1 (6.7% and 5.5% respectively) were not statistically different from each other (Fig.3). The three accessions showed significant ($p \leq 0.05$) differences as regards frequency of callus formed.

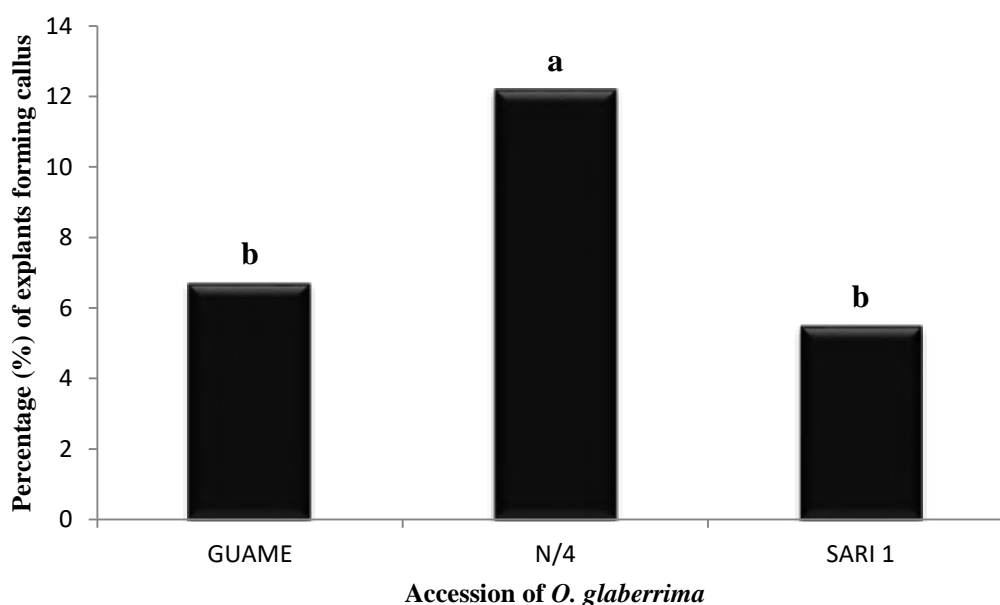


Fig.3: Effect of different accessions of *O. glaberrima* on percentage callus formation (Bars having the same letter are not significantly different ($P \geq 0.05$)).

3.3 Interaction effect of *O. glaberrima* accession and level of 2,4-D in culture medium on callus formation from leaf segments

Generally, callus formation was low even with 2,4-D in the culture medium. However, some accessions performed better than others. Amongst the three *O. glaberrima* accessions, leaf segment explants from N/4 developed the most calli on a medium with 6.0 mg/l 2, 4-D, (40%), with Guame and SARI 1 both recording 20.0% (Fig.4) at the

same concentration of 2,4-D. In all the three rice accessions, percentage callus formation increased with increasing concentration of 2,4-D from 4 mg/l to a peak at 6 mg/l (Fig.4) before dropping.. However, the increase was at different rates depending on the accession. The interaction between accession and concentration of 2,4-D in callus induction medium was however, not statistically significant ($P \geq 0.05$).

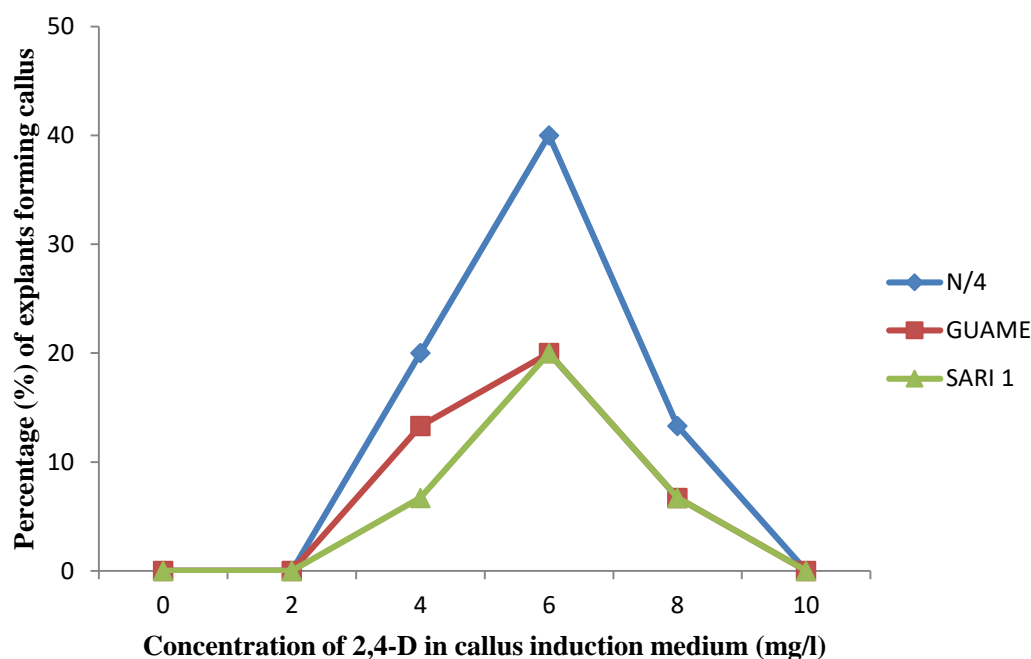


Fig.4: Effect of different accessions of *O. glaberrima* and increasing concentration of 2,4-D in callus induction medium on percentage callus formation

3.4 Effect of NAA and BAP on shoot and root formation from leaf-derived callus of *O. glaberrima*

Calli induced from leaves of the three *O. glaberrima* accessions were further cultured *in vitro* for assessment of shoot and root regeneration ability on MS medium supplemented with a single concentration of NAA (1 mg/l) and varying concentrations (0-5 mg/l) of BAP. Of the six regeneration media evaluated with different combinations of NAA and BAP, none was able to regenerate plantlets (shoots with roots). Instead media containing low levels (0-2 mg/l) of BAP led to prolific root formation from calli while media containing high levels (3-5 mg/l) of BAP led to necrosis of calli. There were no signs of plantlet regeneration from calli sub-cultured onto any of the regeneration media for any of the three *O. glaberrima* accessions.

IV. DISCUSSION

4.1 Effect of hormone type and hormone concentration on callus induction from leaf segments of *O. glaberrima*

Results from the experiment indicate that 2,4-D but not picloram can induce callus from leaf base segments of *O. glaberrima*. Also, 2,4-D at concentrations of 4 mg/l, 6 mg/l and 8 mg/l yielded calli for all three accessions of *O. glaberrima* tested with the best result recorded at 6 mg/l, suggesting that the hormone 2,4-D plays a crucial role in callus induction as earlier reported by Chen *et al.*, (1991). This observation also concurs with previous studies by Ramesh *et al.*, (2009), which showed that 2,4-D at 3.0 mg/l was effective in inducing callus from leaf base segments of *O. sativa*. It is also consistent with findings by Abe and Futsuhara, (1984) who induced embryogenic callus from the roots of *O. sativa* at a concentration of 2.0 mg/l of 2,4-D.

Furthermore, this study revealed that different portions of the leaf responded differently to callus induction with the leaf base segment showing the highest percentage callus induction in all the accessions used for the study. This is consistent with an earlier report by Ramesh *et al.*, (2009) which stated that induction of embryogenic calli from rice leaf is restricted to only the leaf base.

Another interesting observation from this study was the colour of calli obtained. Calli induced from media that had lower levels of 2,4-D were creamish. However, the colour intensified as the concentration of 2,4-D in the induction medium increased. In a related study in chick pea (*Cicerarietinum*L.), by Zamanet *al.*, (2010) calli was induced on MS medium supplemented with varying concentrations of 2,4-D. The colour of these calli were found to be creamish on media containing lower concentrations (0.5 and 1.0) mg/l of 2,4-D, but as concentration increased (1.5 and 2.0) mg/l, creamish brown colour of calli was observed and the highest concentrations (2.5 and 3.0) mg/l gave brownish calli. Zamanet *al.*, (2010) explained that browning of calli occurred if 2, 4-D concentration increases beyond the optimum. Browning of calli was also observed with increased concentration of 2,4-D beyond the optimum by Ramesh *et al.*, (2009), who worked on *indica* rice.

The results of this study also showed that the presence of 2,4-D in culture medium is vital for the induction of callus from leaves of *O. glaberrima*. Absence of 2,4-D resulted in no callus formation among the tested accessions. In most tissue culture experiments, a high auxin/cytokinin ratio is used for initiating embryogenic callus formation compared to a low ratio for the regeneration of plantlets (Geet *al.*, 2006). The exact molecular function of plant growth regulators in tissue culture is unclear. However, it may probably be involved in the reprogramming of the expression of embryogenic genes (Geet *al.*, 2006). In the current study, absence of or very low concentrations of 2,4-D (0-2 mg/l) in the medium did not yield any callus. This observation might be because the concentration of 2,4-D was below level required to trigger cell proliferation. Similarly, at very high concentrations of 2,4-D (10 mg/l) callus failed to form. The inhibition of callus induction may be attributed to phyto-toxic effect of this synthetic auxin on *O. glaberrima*. Significant differences in callus induction were detected among the cultivars when different concentrations of 2,4-D were used.

4.2 Effect of accession of *O. glaberrima* on callus induction from leaf segments

This study proved that the ability to induce callus was greatly influenced by the genotype of *O. glaberrima* used.

The accession N/4 formed the most calli compared to the other two accessions. This demonstrates that different accessions responded differently to callus formation and is in agreement with earlier reports by Gandonouet *et al.* (2005). Significant differences observed in the rates of callus formation of the three accessions of *O. glaberrima* even when subjected to the same nutritional and culture conditions further indicates that callus induction potential is genotype dependent. The differences between the accessions in their response suggest that callus induction may be genetically controlled as observed by some earlier workers (Li *et al.*, 2007; Ozawa *et al.*, 2003; Taguchi-Shiobara *et al.*, 1997).

4.3 Effect of interaction of *O. glaberrima* accession and 2,4-D concentration on callus induction medium on callus formation from leaf segments

The present investigation revealed that all three *O. glaberrima* accessions, concentration of 2,4-D as well as their interaction largely affected callus induction. This observation is in agreement with findings by Pandey *et al.*, (1994), who reported that the success of *in vitro* culture largely depends on the nutritional media, growth regulators, genotype and on the interaction of genotype and medium. Similar reports were also made by earlier workers (Abe and Futsuhara, 1986; Guo and Cao, 1982).

4.4 Effect of NAA and BAP on plantlet regeneration from leaf-derived callus of *O. glaberrima*

Results from the current investigation indicated that the response of the three accessions of *O. glaberrima* to a combination treatment of a single concentration of NAA and different levels of BAP on plantlet regeneration was very poor. The best results obtained from this combination were production of roots at low levels of BAP. This contradicts findings by Ramesh *et al.*, (2009), Ramesh and Gupta, (2005), Boissot *et al.*, (1990) and Abdullah *et al.*, (1986) who reported on the stimulatory effect of BAP in combination with NAA in facilitating plantlet regeneration in rice callus cultures. Differences in response may be due to different concentrations of hormone used.

The rationale behind combining NAA and BAP was to simultaneously regenerate shoots with roots so as to reduce duration of culture of plantlets. NAA is a synthetic auxin that stimulates cell division in the pericycle leading to the formation of lateral and adventitious roots (Taiz and Zeiger, 1998) and BAP is a first-generation synthetic cytokinin that elicits plant growth and development responses, by stimulating cell division (Raven *et al.*, 1999). The

combination of these two hormones obviously did not achieve the intended purpose.

V. CONCLUSIONS

In vitro culture holds potential for improving overall yield of *Oryza glaberrima* Steud., the traditional rice of African origin, with multiple adaptation to several biotic and abiotic stress factors. Based on the results this study, Murashige and Skoog (MS) basal medium supplemented with 30 g/l and 100 mg/l myo-inositol with pH adjusted to 5.8 and incorporated with the plant hormone (auxin) 2,4-dichlorophenoxyacetic acid (2,4-D) at an optimal level of 6 mg/l provided a viable medium for induction of callus in three accessions of *O. glaberrima*, namely N/4, Guame and SARI 1, using leaf base segments as explants. Whole plantlet (shoot with roots) regeneration which was not successful in this study may be achieved through sub-culture of induced calli onto same MS-supplemented medium incorporated with varying combinations of the cytokinin benzy-aminopurine (BAP) and the auxin naphthaleneacetic acid (NAA) to stimulate shoot and root development respectively.

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